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in PPARy Mediated Treatment of Breast Cancer

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13. ABSTRACT (Maximum 200 Words)
Cyclopentenone prostaglandin derivatives of arachidonic acid are promising molecules in the fight against cancer, but their mechanism of action is not well understood. Several investigators have shown that the terminal derivative of prostaglandin J_2 (PGJ_2) metabolism 15deoxy$\Delta^{12,14}PGJ_2$ ($15dPGJ_2$) is a potent activator of the nuclear hormone receptor peroxisome proliferator activated receptor gamma (PPARγ), but $15dPGJ_2$ effects can be mediated by PPARγ-dependent and PPARγ-independent mechanisms. A candidate PPARγ independent mechanism is $15dPGJ_2$ induced inhibition of NFκB *via* covalent modification of IKK, IκBα and the DNA binding domain of NFκB. We have shown previously that $15dPGJ_2$ potently induces apoptosis of breast cancer cells and that $15dPGJ_2$ regulates gene expression critical to apoptosis. Specifically, $15dPGJ_2$ induces potent and irreversible S-phase arrest that is correlated with >2-fold increased expression of at least 20 of 1,176 genes as determined by cDNA differential display. Inhibition of RNA synthesis, using actinomycin D, or protein synthesis, using cycloheximide, abrogates apoptosis induced by $15dPGJ_2$ in breast cancer cells. Additionally, caspase-3 activation follows the induction of gene transcription and the peptide inhibitor ZVAD-fmk blocks apoptosis. These data show that *de novo* gene transcription is necessary for $15dPGJ_2$ induced apoptosis in breast cancer cells, that inhibition of NFκB plays a minor role in $15dPGJ_2$ induced apoptosis and identifies cyclopentenone prostaglandins and potential therapeutic molecules for PPARγ mediated apoptosis in breast cancer.

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Introduction

The peroxisome proliferator activated receptor gamma (PPARγ), is a potential therapeutic target for the treatment of breast cancer but the endogenous ligand for PPARγ is not yet known. Recent data suggest that the endogenous ligand of PPARγ may be a bioactive metabolite of arachidonic acid that is synthesized in normal breast tissue. Activation of PPARγ with different agonists (e.g. 15deoxyΔ12,14PGJ$_2$, troglitazone) elicits different physiological responses in breast cancer cells (i.e. differentiation or apoptosis) raising questions of the role PPARγ plays in normal breast cell physiology. Results from our initial experiments show that prostaglandin metabolites of arachidonic acid inhibit cell cycle progression of MDA-MB-231 breast cancer cells. This cell cycle block induces apoptosis of breast cancer cells and inhibits tumor formation in nude mice. We hypothesize that human breast cancer cell lines (and human breast cancer tumors) have aberrant PPARγ mediated signal transduction pathways or contain disrupted pathways for the metabolism of fatty acid derivatives that act as PPARγ agonists. Understanding the metabolism of fatty acids in breast cancer cells, and elucidating the molecular and signal transduction events that are mediated by PPARγ agonists may lead to novel strategies for the prevention and treatment of breast cancer.

Body

There is extensive literature on the use of retinoic acid and its derivatives, acting through their receptors (RAR and RXR), to arrest or reverse cancer in both animals and humans. Another member of the nuclear receptor superfamily, peroxisome proliferator activated receptor-gamma (PPARγ), has an important role in fat metabolism and adipocyte differentiation. Although its natural ligand is not yet known, synthetic thiazolidinediones, certain fatty acids and metabolites of arachidonic acid, activate PPARγ. Recent data reveal that PPARγ is expressed in colonic tumors and metastatic breast adenocarcinomas, which raises the critical question of its functional significance in human cancers. RXRα and PPARγ agonists together have been shown to induce apoptosis of estrogen receptor positive breast cancer cell lines *in vitro* and attenuate tumor growth in mice. Our studies show that prostaglandin agonists of PPARγ alone inhibit cell cycle progression of both estrogen receptor positive and negative breast cancer cell lines via apoptosis and inhibit tumor formation in nude mice.

There are three specific aims for the pre-doctoral research hypothesis that human breast cancer cell lines (and human breast cancer tumors) have aberrant PPARγ mediated signal transduction pathways or contain disrupted pathways for the metabolism of fatty acid derivatives that act as PPARγ agonists.

1) The first aim is to determine the physiologic activities of different PPARγ agonists on the proliferation of human breast cancer cell lines and primary human breast cancer cells. We will extend our published findings to include other natural prostanoid and eicosanoid agonists (e.g. PGE_2, DHA), synthetic PPARγ agonists (e.g. BRL49653, ciglitazone) and co-activators that can potentiate the effects of PPARγ agonists (e.g. 9-*cis*-retinoic acid, all-*trans*-retinoic acid).

2) The second aim is to determine the molecular mechanisms and signal transduction events that underlie PPARγ mediated differentiation or apoptosis in breast cancer cells.

3) The third aim is to determine the metabolism of J-series prostaglandins in normal breast tissue and breast cancer cells.

Figure 1. AA metabolites activate PPARγ, but the terminal derivative of PGJ_2 metabolism remains the most potent naturally occurring metabolite tested to date.

Aim 1: Our studies of other natural and synthetic PPARγ agonists show that several arachidonic acid (AA) metabolites, including 5- and 15-HETEs and 5-and 15-oxo-EETs, are activators of PPARγ. However, of all the naturally occurring metabolites tested, the terminal derivative of prostaglandin D_2 metabolism, $15deoxy\Delta^{12,14}PGJ_2$ ($15dPGJ_2$), remains the most potent (Figure 1). In addition, after attending the PPARs Keystone Symposium in February 2000, Mr. Clay was successful in obtaining a chemically synthesized selective PPARγ agonist (GW347845X) from GlaxoSmithKlein (GSK). This compound was shown to be 10,000 fold more potent in inducing PPARγ activation by luciferase reporter assays. Although Mr. Clay received this compound only recently, he has confirmed the reports by GSK and will add this compound to his arsenal of PPARγ agonists in determining the physiologic activity of these compounds in breast cancer cell lines. A major accomplishment of Mr. Clay's was his observation that the published literature cites different physiologic outcomes in various cancer cell lines according to the concentration of PPARγ agonist used. To this end, Mr. Clay authored a review article that documented the differing biological effects of PPARγ activation in diverse cell types (1). Furthermore, Mr. Clay undertook the responsibility of determining if these diverse and opposing biologic outcomes occur in a single cell type (2). Mr. Clay will investigate if agonists of RXRα, the heterodimeric partner of PPARγ, could potentiate the observed responses.
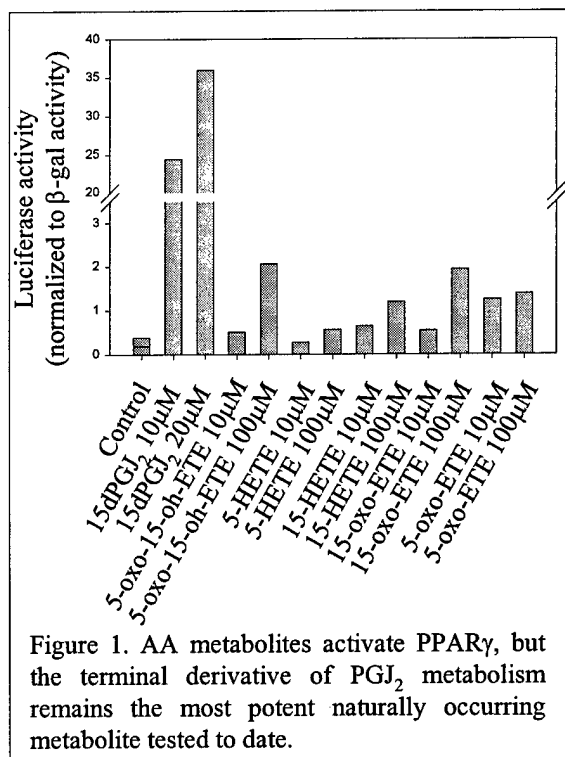
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Aim 2: The molecular mechanisms and signal transduction events that underlie PPARγ mediated differentiation or apoptosis in breast cancer cells are complex and not well understood. Mr. Clay has achieved great milestones in elucidating parts of these pathways. In a screen of 1,176 gene produts by cDNA array analysis, Mr. Clay identified particular gene products that are increased in breast cancer cell lines after treatment with 15dPGJ$_2$. Of these, the expression of the cyclin dependent kinase inhibitors p21$^{Waf1/Cip1}$ (p21) and p27^{Kip1} (p27) and the cyclins D and E is increased >2 fold. Additionally, the expression of several genes involved in DNA maintenance and repair is decreased >2 fold. Mr. Clay has performed *post hoc* analysis of p21 and p27 expression by Western blot analysis to confirm the results from the cDNA array (Figure 2) and will establish cell lines that express a dominant negative form of p21. Additionally, Mr. Clay has followed up on published reports of the effects of 15dPGJ$_2$ in other cell systems to devise a potential mechanism by which 15dPGJ$_2$, or other cyclopentenone prostaglandins, may exert such potent anti-neoplastic activity in a variety of cancer cell types (Appendix 1). These studies have resulted in the preparation of a manuscript that Mr. Clay intends to submit to *The Journal of Biological Chemistry* (3). Mr. Clay will continue this line of investigation to include other gene products and further elucidate the mechanisms described. Furthermore, Mr. Clay has established breast cancer cell lines that express a dominant negative form of PPARγ. He has shown that
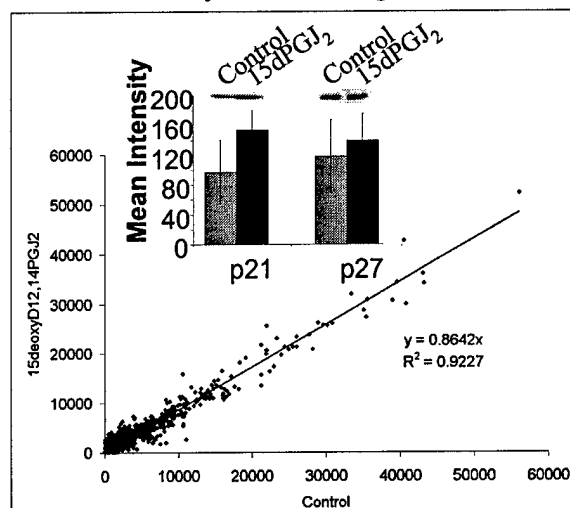

Figure 2. cDNA array analysis of 1,176 genes shows that the the majority of expressed gene do not change in response to treatment with 15dPGJ$_2$. The mRNA expression of cyclin dependent kinases p21 and p27 was increased >2 fold. Expression of p21 and p27 protein was confirmed by Western blot analysis.

transcriptional activation of PPARγ by 15dPGJ$_2$ is blocked in these cells (Figure 3) and will continue to investigate how the dominant negative cell lines affect apoptosis induced by 15dPGJ$_2$. Recent publications suggest that 15dPGJ$_2$ negatively regulates the NFκB pathway of gene transcription. Mr. Clay has begun to investigate this critical pathway using NFκB inhibitors, Bay and Cape, and by establishing cell lines that express a dominant negative form of the NFκB regulator IκBα.

Aim 3: The studies of the metabolism of J-series prostaglandins in normal breast tissue and breast cancer cells are in the beginning stages. Mr. Clay was successful in obtaining a small amount of [^{3}H]15dPGJ$_2$ through a collaborative effort with Dr. Kirk Maxey of Cayman Chemical. Using [^{3}H]15dPGJ$_2$ to follow the metabolism of 15dPGJ$_2$ in the breast cancer cell line MDA-MB-231, Mr. Clay has noted that after 12 hours, the majority of label is still present as 15dPGJ$_2$. In this preliminary study, 66% of [^{3}H]15dPGJ$_2$ was recovered after 12 hours. The remaining 44% was in the form of more polar metabolites as determined by thin layer chromatography (TLC). These derivative may represent a class of reactive oxygen species (ROS) that further activate PPARγ (Appendix 1). Mr. Clay was unable to determine the structure of these polar metabolites, or their biological activity, due to the limited quantity of material, but Mr. Clay has enlisted the analytical expertise of the laboratory of Dr. Robert Murphy (National Jewish Research Center, Denver, Colorado) to assist with the determination of these structures by negative ion chemical ionization gas chromatography/tandem mass spectrometry (NICI GC/MS/MS). Moreover, Mr. Clay has obtained critical reagents for the study of prostaglandin metabolism. Specifically, Mr. Clay has been promised the use of immuno-reactive antibodies to specific AA metabolizing enzymes. These include antibodies to fatty acid CoA ligase (FACL4), the enzyme that ligates free AA to Co-enzyme A, cyclooxygenase 2 (COX-2), the enzyme which catalyzes the oxidation and cyclization of AA to produce prostaglandin G$_2$ (PGG$_2$) and prostaglandin H$_2$ (PGH$_2$) and prostaglandin D$_2$ synthase (PGDS), the enzyme that catalyzes the formation of PGD$_2$ from PGG$_2$/PGH$_2$. These reagents will be helpful for the investigation of enzymatic levels of these critical metabolizing enzymes. In addition, enzymatic activity assay kits are readily available.

Key Research Accomplishments

- $15\text{deoxy}\Delta^{12,14}\text{PGJ}_2$ remains the most potent naturally occurring PPARγ agonist identified.
- The degree of PPARγ activation dictates distinct and opposing biological responses in breast cancer cells, ranging form increased proliferation to differentiation and apoptosis.
- $15\text{deoxy}\Delta^{12,14}\text{PGJ}_2$ induced apoptosis requires *de novo* expression of critical gene products.
- Dominant negative expression of PPARγ completely abrogates transcriptional activation induced by $15\text{deoxy}\Delta^{12,14}\text{PGJ}_2$.
- The mechanism of action of $15\text{deoxy}\Delta^{12,14}\text{PGJ}_2$ is not limited to PPARγ activation. $15\text{deoxy}\Delta^{12,14}\text{PGJ}_2$ can inhibit NFκB, activate PPARγ and can stimulate reactive oxygen species generation. Together, these events lead to induced expression of key gene products that are involved in PPARγ mediated apoptosis in breast cancer cells.
- $15\text{deoxy}\Delta^{12,14}\text{PGJ}_2$ is metabolized to polar derivatives by breast cancer cells.

Figure 3. Expression of dominant negative PPARγ blocks transcriptional activation in duced by 15dPGJ₂.

Reportable Outcomes

- Manuscripts
    1. **Clay CE**, Namen AM, Fonteh AN, Atsumi G, High KP, Chilton FH, 2000, 15deoxyΔ12,14PGJ₂ induces diverse biological responses via PPARγ activation in cancer cells. *Prostaglandins and Other Lipid Mediators* 62:23-32
    2. **Clay CE**, Namen AM, Atsumi G, Trimboli AJ, Fonteh AN, High KP, Chilton FH, 2001, The magnitude of PPARγ activation is associated with important and seemingly opposite biological responses in breast cancer cells. Journal of Investigational Medicine (in press)
    3. **Clay CE**, Atsumi G, High KP, Chilton FH. 2001, 15deoxyΔ^{12,14}PGJ₂ induced apoptosis is not mediated by NFκB in breast cancer cells: requirement for *de novo* gene expression. (in preparation)
    4. **Clay CE**, Atsumi G, High KP, Chilton FH. 2001, PPARγ dependent and independent mechanisms of apoptosis in breast cancer cells (in preparation)

- Abstracts
    1. *PPARγ induced apoptosis requires de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* FASEB: Receptors and Signal Transduction, Copper Mountain, CO July 2-9, 2000

    2. *15deoxyΔ^{12,14}PGJ₂ inhibits breast cancer cell proliferation via PPARγ activation.* International Society for Preventive Oncology, 5th International Meeting, Geneva, Switzerland, October 28-31, 2000, Satellite Symposium October 29, 2000

    3. *PPARγ induced apoptosis requires de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University, Breast Cancer Center of Excellence, Winston Salem, NC, November 16, 2000

    4. *PPARγ induced apoptosis requires de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Keystone Symposium: PPARs a transcription odyssey, Keystone, CO, February 2-9, 2001
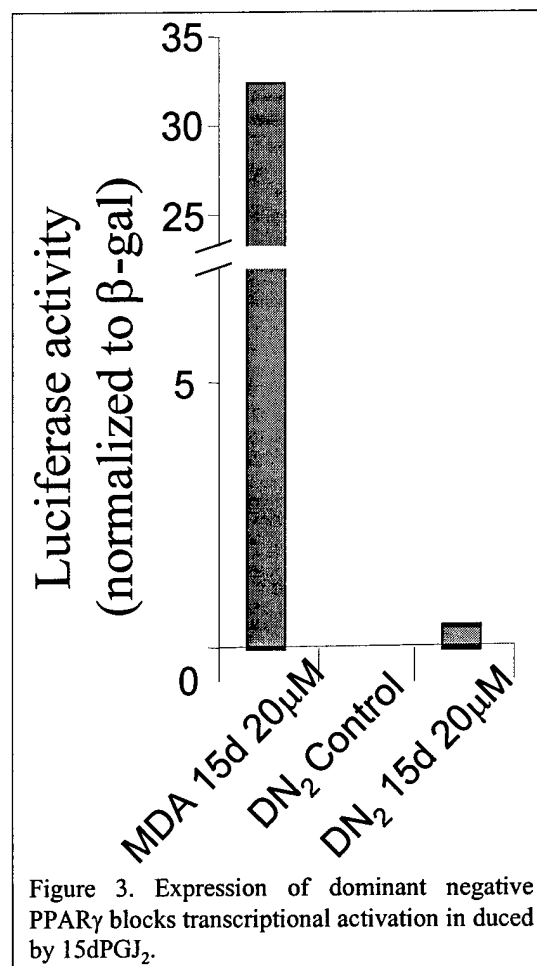
- Presentations
  1. *PPARγ induced biologic responses require de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University Cancer Center Faculty Retreat, Winston-Salem, NC, August 11-12, 2000

  2. *15deoxyΔ^{12,14}PGJ$_2$ induced apoptosis in suppressed by a PPARγ dominant negative.* South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

  3. *Mechanisms of Apoptosis in breast cancer cells: 15deoxyΔ^{12,14}PGJ$_2$ and PPARγ.* University of Colorado Health Sciences Center, Denver, CO, February 9, 2001.

- Development of cell lines
  1. PPARγ Dominant Negative
  2. IκBα Dominant Negative
  3. p21 Dominant Negative

- Awards
  1. Comprehensive Cancer Center Award: Best graduate student presentation (monetary award) *PPARγ induced biologic changes require de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University Cancer Center Faculty Retreat, August 11-12, 2000

  2. Avanti Founder's Award: Best graduate student presentation (monetary award and conference expenses) *15deoxyΔ^{12,14}PGJ$_2$ induced apoptosis in suppressed by a PPARγ dominant negative.* South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

- Funding applied for based on work supported by this award
  1. Susan G. Komen Breast Cancer Foundation Dissertation Award. *PPARγ Induced Apoptosis Requires de novo Gene Expression in Breast Cancer Cells: searching for key molecular targets.* (submitted March 15, 2001)

  2. Wake Forest University Comprehensive Cancer Center. *PPARγ and soy phytoestrogens as possible therapy for breast cancer.* $10,000 (submitted March 15, 2001)

Conclusions

Naturally occurring derivatives of arachidonic acid metabolism are potent and effective activators of PPARγ. The most potent of these derivatives is 15deoxyΔ^{12,14}PGJ$_2$ (15dPGJ$_2$), the dehydration and isomerization product of prostaglandin D$_2$ (PGD$_2$). 15dPGJ$_2$ induces PPARγ mediated transcriptional activation leading to the synthesis of critical gene products involved in cell cycle arrest and apoptosis. Of these gene products, expression of the cyclin dependent kinase inhibitors, p21 and p27, is associated with marked cell cycle arrest with subsequent apoptosis involving caspase-3. Although 15dPGJ$_2$ inhibits NFκB mediated transcription, this likely represents a minor contribution to 15dPGJ$_2$ induced apoptosis in breast cancer cells. Investigations into altered fatty acid metabolism pathways are underway and may yield clues as to how arachidonic acid derivative exert such potent anti-neoplastic activity in breast cancer cells. 15dPGJ$_2$ may represent a novel class of therapeutic molecules for the PPARγ mediated treatment of breast cancer.

References

1. **Clay CE,** Namen AM, Fonteh AN, Atsumi G, High KP, Chilton FH, 2000, 15deoxyΔ12,14PGJ$_2$ induces diverse biological responses via PPARγ activation in cancer cells. *Prostaglandins and Other Lipid Mediators* 62:23-32
2. **Clay CE,** Namen AM, Atsumi G, Trimboli AJ, Fonteh AN, High KP, Chilton FH, 2001, The magnitude of PPARγ activation is associated with important and seemingly opposite biological responses in breast cancer cells. *Journal of Investigational Medicine* (in press)

Appendices

Appendix 1: **Mechanisms of 15deoxy$\Delta^{12,14}$PGJ$_2$ induces apoptosis in breast cancer cells.** 15dPGJ$_2$ induced apoptosis in breast cancer cells involves the expression of critical gene products, such as p21 and p27, via the activation of PPARγ. NFκB signaling represents a minor contribution, if any, to 15dPGJ$_2$ induced apoptosis in breast cancer cells. Activation of phospholipases (PLA$_2$) and inhibitors of AA metabolism, such as NSAIDs, triacsin C and CoA-IT inhibitors, increase free AA levels. 15dPGJ$_2$ induced reactive oxygen species (ROS) that oxidize arachidonic acid may generate oxidized lipid products that may further activate PPARγ.

$$15deoxy\Delta^{12,14}PGJ_2$$

or other
cyclopentenone
prostaglandins